

Research report

Regional influence of cocaine on evoked dopamine release in the nucleus accumbens core: A role for the caudal brainstem

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ABSTRACT

Cocaine increases dopamine concentration in the nucleus accumbens through competitive binding to the dopamine transporter (DAT). However, it also increases the frequency of dopamine release events, a finding that cannot be explained by action at the DAT alone. Rather, this effect may be mediated by cocaine-induced modulation of brain regions that project to dopamine neurons. To explore regional contributions of cocaine to dopamine signaling, we administered cocaine to the lateral or fourth ventricles and compared the effects on dopamine release in the nucleus accumbens evoked by electrical stimulation of the ventral tegmental area to that of systemically-delivered cocaine. Stimulation trains caused a sharp rise in dopamine followed by a slower return to baseline. The magnitude of dopamine release ([DA]max) as well as the latency to decay to fifty percent of the maximum ($t(1/2)$; index of DAT activity) by each stimulation train were recorded. All routes of cocaine delivery caused an increase in [DA]max; only systemic cocaine caused an increase in $t(1/2)$. Importantly, these data are the first to show that hindbrain (fourth ventricle)-delivered cocaine modulates phasic dopamine signaling. Fourth ventricular cocaine robustly increased cFos immunoreactivity in the nucleus of the solitary tract (NTS), suggesting a neural substrate for hindbrain cocaine-mediated effects on [DA]max. Together, the data demonstrate that cocaine-induced effects on phasic dopamine signaling are mediated via actions throughout the brain including the hindbrain.

1. Introduction

The development of drug addiction begins with the initial reinforcing effects of drugs that involve brain dopamine. While different classes of addictive drugs vary in their primary pharmacological mechanisms (Luscher and Ungless, 2006), they all share an acute effect of increasing dopamine concentration in the striatum, particularly in the nucleus accumbens (NAc) (Di Chiara and Imperato, 1988; Cheer et al., 2007), an effect essential for reinforcement. Continued use of addictive substances and therefore enhanced dopaminergic signaling can lead to physiological changes within the striatum that contribute to the perseverant conditioned responses seen in drug addiction. The psychostimulant cocaine exerts its reinforcing effects through its actions in the central nervous system. Indeed, rats generalize the effects of systemic cocaine to central nervous system delivery (Wood et al., 1987) and will self-administer cocaine directly to dopamine terminals regions (McBride et al., 1999). Cocaine must also enter the central nervous system to evoke dopamine signaling in the NAc

(Porter-Stransky et al., 2011). The primary mechanism of cocaine action on NAc dopamine concentration is through its ability to block the reuptake of dopamine by the dopamine transporter (DAT) (Giros et al., 1996; McElvain and Schenk, 1992). Aspects of cocaine reward are dependent on its binding to the DAT (Chen et al., 2006). Consistent with this mechanism, systemic cocaine increases the magnitude and decreases the decay rate of electrically-evoked, phasic increases in extracellular dopamine (Wu et al., 2001).

Systemic cocaine also increases the magnitude and duration of spontaneously occurring dopamine release events termed ‘transients’ (Heien et al., 2005; Aragona et al., 2008). Interestingly, it also increases transient frequency - a result that would not be predicted based on reuptake blockade alone (Aragona et al., 2008; Covey et al., 2014). These transients can become time-locked to reward predictive stimuli (Stuber et al., 2008; Aragona et al., 2009) and are correlated with conditioned approach to reward predictive cues (Stuber et al., 2008; Cone et al., 2016; Aragona et al., 2009). They are thought to be critical for incentivizing goal-directed behavior (Hamid et al., 2016)

Abbreviations: DAT, dopamine transporter; [DA]max, maximal evoked dopamine release; FG, fluorogold; FSCV, fast-scan cyclic voltammetry; GLP-1, glucagon-like-peptide 1; NAc, nucleus accumbens; NE, norepinephrine; NMDA, N-methyl-D-aspartate

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and thus play a critical role in the development of addictive behaviors. Dopamine transients induced by cocaine are linked to the excitability of dopamine neurons, as inactivation of the ventral tegmental area (VTA; the site of dopamine cell bodies projecting to the NAc) by lidocaine abolishes cocaine-induced NAc transient activity (Aragona et al., 2008; Owesson-White et al., 2012). Furthermore, recent work has suggested that cocaine enhances excitatory inputs to dopamine neurons. Cocaine increases the firing rate of VTA dopamine neurons following cocaine administration (Mejias-Aponte et al., 2015). In addition, phasic dopamine release caused by intravenous cocaine is dependent on excitatory noradrenergic receptors in the VTA (Goertz et al., 2015). Thus, accumulating evidence suggests that cocaine increases excitatory drive to dopamine neurons. However, VTA dopamine neurons receive inputs from cocaine-sensitive sites throughout the neuraxis and it remains unclear which regions of the brain affected by cocaine delivery contribute to the increase in the excitability of dopamine neurons and NAc dopamine release.

To explore regionally selective actions of cocaine on phasic dopamine signaling in the NAc, we delivered cocaine to either the lateral or fourth ventricle at a dose that establishes a conditioned place preference (Morency and Beninger, 1986) and generalizes to a self-administered systemic dose when delivered to the lateral ventricle (Wood et al., 1987). We sampled dopamine concentration in the NAc using fast-scan cyclic voltammetry (FSCV) and compared the effects of centrally-administered cocaine on dopamine release electrically-evoked by stimulation of the VTA to those of systemic delivery. We measured the magnitude of electrically-evoked dopamine release [which is influenced by both dopamine neuron excitability (Van Zessen et al., 2012) and the rate of dopamine reuptake] and the latency for dopamine to decay from peak concentration (an index of reuptake (Yorgason et al., 2011)). Additionally, immunohistochemistry was performed to determine if VTA projections from the caudal brainstem could contribute to effects of fourth ventricular cocaine on dopamine signaling. Collectively, the results support the hypothesis that cocaine acts widely throughout the central nervous system to influence dopamine signaling through multiple mechanisms but also point to a

surprising role for the caudal brainstem.

2. Results

For FSCV experiments, histological analyses indicated that all measurements were made in the NAc core (Fig. 1A). Data were normalized to average of the 3 baseline measurements and expressed as percent change for all subjects. It should be noted, though, that there were no differences between experimental groups in any experiment prior to pharmacological manipulations. In addition, we confirmed that infusion cannula were placed in either the lateral or fourth ventricle (see Fig. 1B and C for representative examples).

2.1. Systemic cocaine potentiates the magnitude and duration of electrically-evoked dopamine release events in the NAc

Cocaine (2.5 mg/kg; IP) reliably increased the magnitude of electrically-evoked dopamine release events in the NAc. The effects of drug treatment on magnitude are represented by Fig. 2A where the average maximal evoked dopamine release ([DA]max) at each of the 23 time points is expressed as percent change from baseline. A two-way ANOVA revealed that the magnitude of electrically-evoked dopamine release varied as a function of time, [$F(1, 23)=3.60, p < 0.001$], as well as a function of drug treatment, [$F(1, 23)=19.45, p < 0.001$]. More importantly, a significant interaction was found such that cocaine-treated rats showed an increase in [DA]max at specific time points relative to saline-treated rats, [$F(1, 23)=9.26, p < 0.001$]. *Post-hoc* Bonferroni-corrected *t*-tests revealed differences between cocaine and saline treatment beginning at 12 min post-drug administration and continuing through the end of the recording period.

Systemic cocaine also reliably increased the duration of electrically-evoked dopamine release events across time. Duration was calculated as the latency for the signal to decay to half maximum ($t(1/2)$) and is expressed as a percent change from baseline in Fig. 2B. The duration of dopamine release events varied both as a function of time, [$F(1, 23)=7.11, p < 0.001$], as well as by drug treatment, [$F(1, 23)=32.02, p < 0.001$].

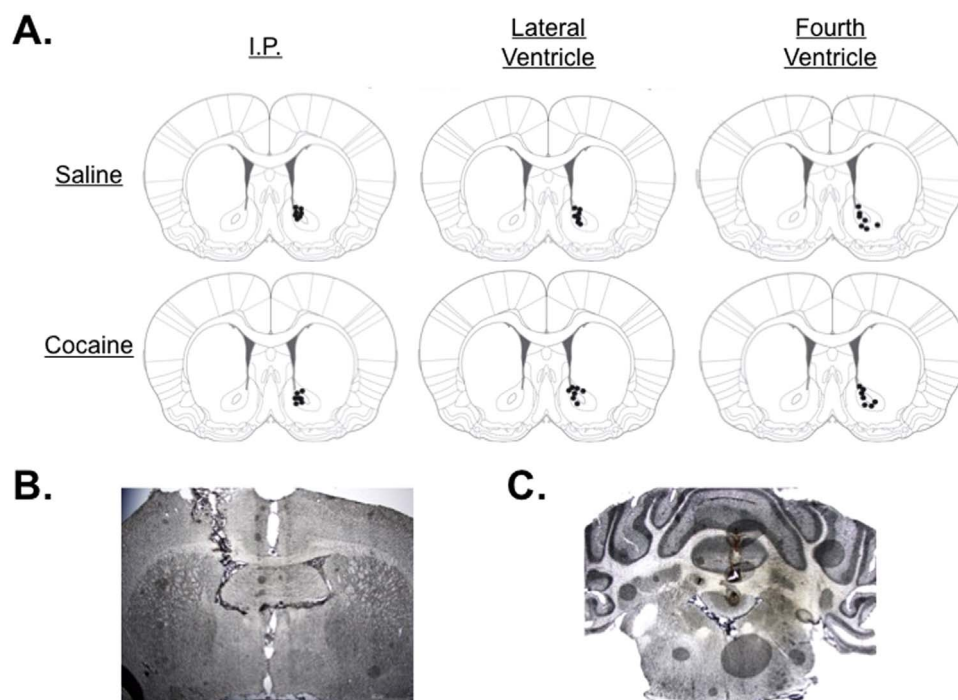


Fig. 1. Histological verification of FSCV recordings and ventricular infusions. (A). Summary of FSCV recording sites in the NAc core categorized by experimental treatment. Black dots indicate the location of the electrolytic lesion created in each animal. (B). Representative image of histological verification of lateral ventricular infusions using India ink. (C). Representative image of histological verification of fourth ventricular infusions using India ink.

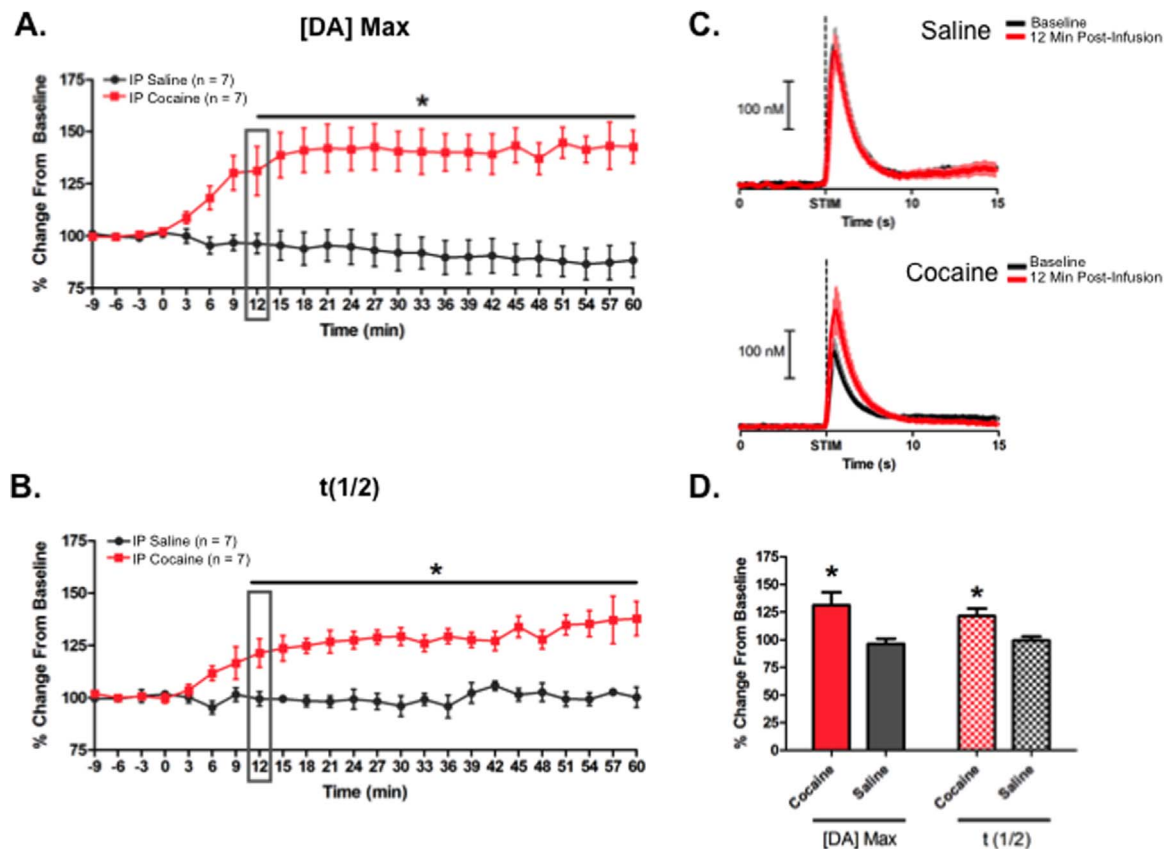


Fig. 2. Systemic cocaine potentiates both magnitude and duration of electrically-evoked dopamine release events in the NAc. (A). Average [DA]max at each of the 23 time points expressed as percent change from baseline. Symbols represent means and error bars represent ± 1 standard error of the mean (SEM) for saline- (black filled circle and line) and cocaine- (red filled squares and line) injected rats. (B). Average latency for the signal to decay to half maximum ($t(1/2)$) expressed as a percentage of baseline. Conventions are identical to those of A. (C). Average change in dopamine concentration evoked during the final baseline stimulation (black trace) is plotted with the average change in dopamine concentration evoked 12 min after injection (red trace) for saline (top) and cocaine (bottom) injected rats. (D). Summary of average [DA] max and $t(1/2)$ at 12 min post-injection expressed as percent change. Bars represent means and error bars represent ± 1 SEM for saline (dark gray) and cocaine (red) treatment.

0.001]. As with magnitude, a significant interaction was found such that cocaine-treated rats showed an increase in duration of release events at specific time points relative to saline-treated rats, [$F(1, 23) = 6.98, p < 0.001$]. *Post-hoc* analyses revealed that differences between cocaine- and saline-injected rats emerged at 12 min ($p < 0.05$) and remained significant through the remainder of the 60 min post-injection session ($p < 0.05$). Since significant differences emerged at 12 min (denoted by rectangle in Fig. 2A and B) following injection, the average dopamine spike evoked during the final baseline stimulation is plotted with the average dopamine spike evoked 12 min after injection for saline- (top) and cocaine- (bottom) injected rats in Fig. 2C. A summary of both magnitude and duration effects at the 12 min time point for both systemic saline and cocaine groups is depicted in Fig. 2D.

2.2. Lateral ventricular cocaine potentiates the magnitude but not duration of electrically-evoked dopamine release events in the NAc

Lateral ventricular cocaine (50 $\mu\text{g}/1 \mu\text{l}$) reliably increased the magnitude of electrically-evoked dopamine release. The average [DA] max at each time point is represented as percent change from baseline for both saline- and cocaine-treated rats in Fig. 3A. Analysis revealed a main effect of both time, [$F(1, 23) = 5.80, p < 0.001$], and drug treatment [$F(1, 23) = 12.50, p < 0.01$]. Additionally, a significant interaction was found where animals receiving lateral ventricular cocaine showed an increase of magnitude across time compared to saline-treated animals, [$F(1, 23) = 4.86, p < 0.001$]. *Post-hoc* analyses revealed this interaction was significant between the 6 min ($p < 0.05$) and 30 min ($p < 0.05$) time points. Therefore lateral ventricular cocaine increases the

magnitude of electrically-evoked dopamine release in NAc in a more rapid, yet shorter-lived manner as compared to systemic cocaine.

Unlike the effects on magnitude, lateral ventricular cocaine failed to significantly affect the duration of electrically-evoked dopamine release events. The average $t(1/2)$ is expressed as percent change from baseline for each time point in Fig. 3B. While statistical analyses revealed a main effect of time, [$F(1, 23) = 4.15, p < 0.001$], there was no significant effect of drug treatment, [$F(1, 23) = 1.34, p = \text{n.s.}$], nor was there an interaction of cocaine exposure across time, [$F(1, 23) = 1.33, p = \text{n.s.}$]. As the effects of systemic cocaine were first significant 12 min after drug administration, this time point was used for all experimental groups to compare differences in both magnitude and duration of electrically-evoked dopamine release events. Average dopamine spikes comparing the evoked change in dopamine concentration during the final baseline sample and 12 min post-treatment from both saline (top) and cocaine (bottom) treated rats are illustrated in Fig. 3C. A summary for both [DA]max, and $t(1/2)$ is represented in Fig. 3D as the average percent change from baseline at 12 min post-treatment for both saline- and cocaine-injected rats.

2.3. Fourth ventricular cocaine potentiates the magnitude but not duration of electrically-evoked dopamine release events in the NA

Fourth ventricular cocaine reliably increased the magnitude of electrically-evoked dopamine release events in the NAc. The effect of cocaine, as compared to saline, on magnitude can be seen in Fig. 4A, where [DA]max is represented as a percent change from baseline across each time point recorded. Statistical analyses revealed a main effect of time, [$F(1, 23) = 6.39, p < 0.001$], as well as a main effect of

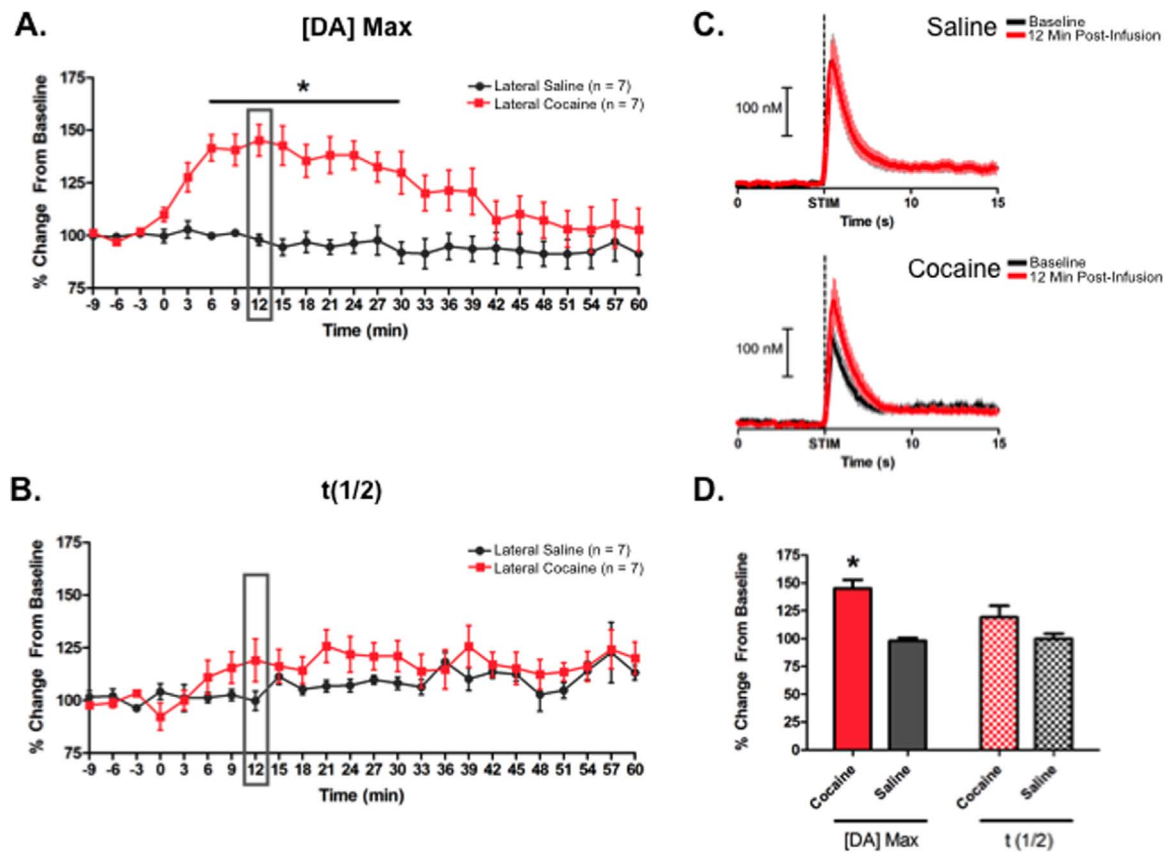


Fig. 3. Lateral ventricular cocaine potentiates the magnitude but not duration of electrically-evoked dopamine release events in the NAc. (A). Average [DA]max at each of the 23 time points and expressed as percent change from baseline. Symbols are the means and error bars represent ± 1 SEM for saline (black filled circle and line) and cocaine (red filled squares and line) injected rats. (B). Average latency for the signal to decay to half maximum ($t(1/2)$) expressed as a percentage of baseline. Conventions are identical to those of Fig. 2A. (C). Average change in dopamine concentration evoked during the final baseline stimulation (black trace) is plotted with the average change in dopamine concentration evoked 12 min after injection (red trace) for saline (top) and cocaine (bottom) injected rats. (D). Summary of average [DA] max and $t(1/2)$ at 12 min post-injection expressed as percent change. Bars represent means and error bars represent ± 1 SEM for saline (dark gray) and cocaine (red) treatment.

drug treatment, [$F(1, 23) = 6.15, p < 0.05$]. These effects were moderated by a significant interaction, [$F(1, 23) = 5.11, p < 0.001$], such that fourth ventricular cocaine increased the magnitude of dopamine release events over time compared to saline-treated animals. *Post-hoc* Bonferroni *t*-tests proved revealed significant differences from 6 min following drug administration to 21 min. Therefore, similar to lateral ventricular cocaine delivery, cocaine administered to the fourth ventricle had a more rapid, yet shorter-lived effect on the magnitude of electrically-evoked dopamine in the NAc compared to systemic cocaine.

Fourth ventricular, like lateral ventricular, cocaine failed to cause a significant effect on the duration of electrically-evoked dopamine release. Average $t(1/2)$ is represented as a percent change from baseline across time in Fig. 4B. Analyses revealed a main effect of time, [$F(1, 23) = 2.52, p < 0.001$] but no significant main effect of drug treatment, [$F(1, 23) = 1.65, p = \text{n.s.}$] or interaction of drug treatment over time on duration, [$F(1, 23) = 0.907, p = \text{n.s.}$]. Thus, while fourth ventricular cocaine significantly modulates the magnitude of electrically-evoked dopamine release in the NAc, it has no significant effect on the duration of such events. Average dopamine spikes for both cocaine- and saline-treated animals at the final baseline recording and 12 min post-drug administration are illustrated in Fig. 4C. Additionally, a summary of both fourth ventricular saline and cocaine effects on [DA] max and $t(1/2)$ at the 12 min time point are represented in Fig. 4D.

2.4. Fourth ventricular cocaine induces cFos in the NTS

To investigate potential inputs to the VTA that might be responsible for the augmented evoked dopamine release following fourth ventricular delivery of cocaine, we triple-labeled cells in the nucleus of the

solitary tract (NTS) for cFos, tyrosine hydroxylase (TH) and VTA-injected fluorogold (FG; a monosynaptic retrograde tracer to identify VTA-projecting cells). The NTS was investigated due to recently identified, monosynaptic connections with the VTA (Alhadeff et al., 2012; Mejias-Aponte et al., 2009) as well as its responsiveness to systemic cocaine (Buffalari and Rinaman, 2014). At each level of the NTS examined (-14.2 , -13.8 , and -13.4 mm posterior to bregma), there were statistically significant increases in cFos immunofluorescence in rats that received fourth ventricular cocaine vs. saline (all p s < 0.05). Fig. 5 provides representative images of FG injection sites in the VTA (Fig. 5A), cFos expression in the NTS (Fig. 5B) as well as co-localization of cFos, TH and FG in the NTS (Fig. 5C) of cocaine- vs. saline-treated rats. Average cell counts and co-localization percentages for each level of the NTS are provided below and in Table 1.

In the NTS at the approximate level of bregma -14.2 mm, there were 5.2 ± 1.0 cFos-expressing cells per unilateral brain section in fourth ventricular cocaine-treated rats compared to 0.11 ± 0.11 cFos-expressing cells in fourth ventricular saline-treated rats ($p < 0.05$). While differences in cFos were apparent, NTS sections did not differ with respect to the number of either TH or FG labeled cells (cocaine- vs. saline-treated rats all p s = ns). In fourth ventricular cocaine-treated rats, $35.9 \pm 8.6\%$ of cFos cells co-expressed TH, $11.9 \pm 7.0\%$ of cFos cells co-expressed FG, and $5.4 \pm 2.5\%$ of cFos cells co-expressed both TH and FG.

In the NTS at approximately bregma -13.8 mm level, there were 13.4 ± 2.2 cFos-expressing cells per unilateral brain section in fourth ventricular cocaine-treated rats compared to 0.33 ± 0.17 cFos-expressing cells in fourth ventricular saline-treated rats ($p < 0.01$). There were no differences with respect to FG- or TH-positive cells (all p s = ns). In

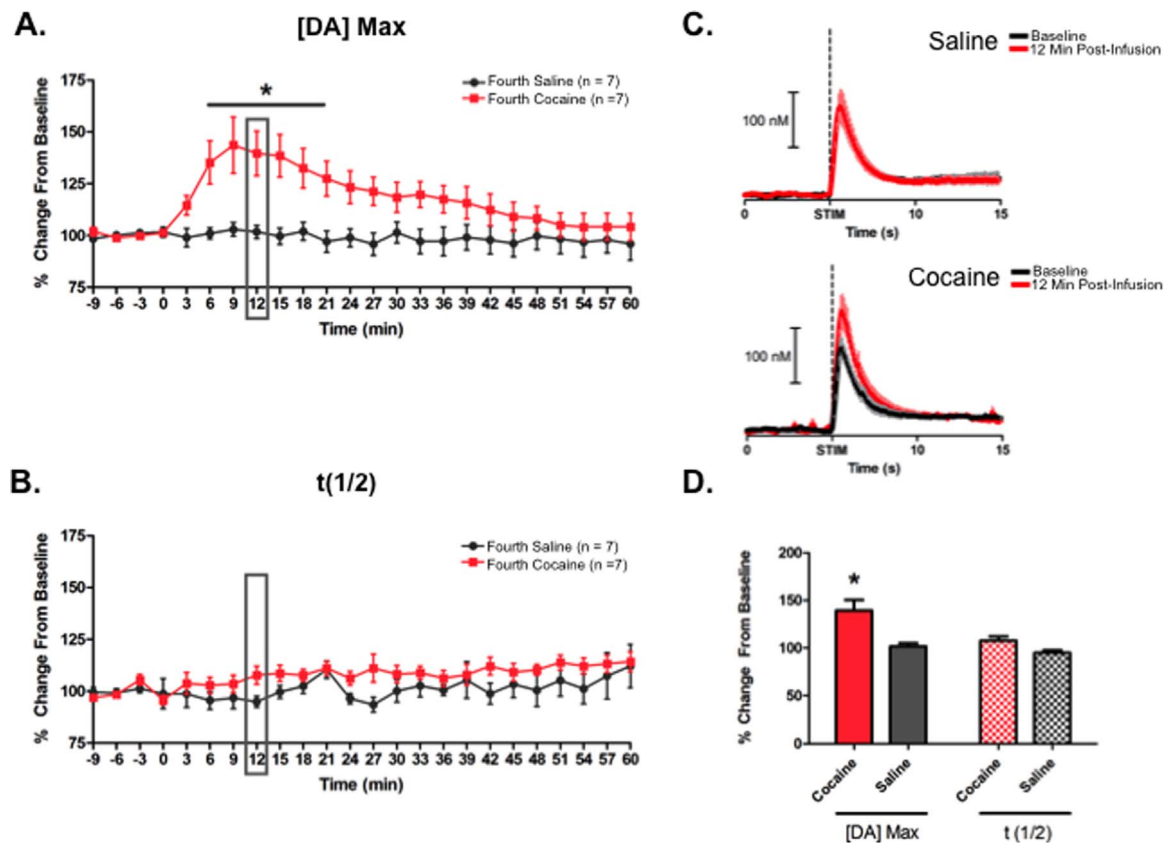


Fig. 4. Fourth ventricular cocaine potentiates the magnitude but not duration of electrically-evoked dopamine release events in the NAc. (A). Average [DA]max at each of the 23 time points and expressed as percent change from baseline. Symbols are the means and error bars represent ± 1 SEM for saline (black filled circle and line) and cocaine (red filled squares and line) injected rats. (B). Average latency for the signal to decay to half maximum ($t(1/2)$) expressed as a percentage of baseline. Conventions are identical to those of Fig. 2A. (C). Average change in dopamine concentration evoked during the final baseline stimulation (black trace) is plotted with the average change in dopamine concentration evoked 12 min after injection (red trace) for saline (top) and cocaine (bottom) injected rats. (D). Summary of average [DA] max and $t(1/2)$ at 12 min post-injection expressed as percent change. Bars represent means and error bars represent ± 1 SEM for saline (dark gray) and cocaine (red) treatment.

fourth ventricular cocaine-treated rats, approximately $43.2 \pm 4.7\%$ of cFos cells co-expressed TH, $5.1 \pm 1.6\%$ of cFos cells co-expressed FG, and $2.6 \pm 0.9\%$ of cFos cells co-expressed both TH and FG.

More anterior in the NTS at approximately bregma -13.4 mm, there were 15.0 ± 3.1 cFos-expressing cells per unilateral brain section in fourth ventricular cocaine-treated rats compared to 1.3 ± 1.1 cFos-expressing cells in fourth ventricular saline-treated rats ($p < 0.05$). There were no differences with respect to FG- or TH-positive cells (all $ps = n.s.$). In fourth ventricular cocaine-treated rats, approximately $34.3 \pm 3.7\%$ of cFos cells co-expressed TH, $3.2 \pm 1.5\%$ of cFos cells co-expressed FG, and $1.9 \pm 0.9\%$ of cFos cells co-expressed both TH and FG.

3. Discussion

Mesolimbic dopamine signaling is essential to the initial reinforcing properties of drugs of abuse and the formation of drug addiction. The primary mechanisms by which different classes of addictive drugs potentiate dopamine signaling have largely been elucidated, however there remain additional effects on dopamine action that cannot be explained by their traditional actions alone. This study examined the effects of cocaine acting in different parts of the neuraxis on NAc dopamine signaling to uncover possible alternative drug targets. We found that cocaine administered to the fourth ventricle – which restricted cocaine action to the hindbrain (Hayes et al., 2009) – potentiated the magnitude ([DA] max) of electrically evoked dopamine release in the NAc. This effect occurred in the absence of an effect on the duration ($t(1/2)$) of evoked release and hence cannot be explained by dopamine reuptake blockade – the traditional explanation for

cocaine action on dopamine signaling. The rapid time course of this effect and its lack of effect on duration of evoked dopamine release events in the NAc were almost identical to that of lateral ventricular cocaine. Both centrally administered effects of cocaine were compared to systemic delivery (2.5 mg/kg) where, not surprisingly, we observed an effect of cocaine on both magnitude and duration of evoked dopamine release events. Systemic cocaine delivery followed a slightly longer onset yet prolonged time course overall.

Previous work has shown that cocaine increases both the magnitude and duration of electrically-evoked dopamine release in the NAc in both slice preparations (Jones et al., 1995a, 1995b) and in vivo when given systemically (Suaud-Chagny et al., 1995; Jones et al., 1995a; Heien et al., 2005). Therefore our results from systemic cocaine exposure were expected, however, they served as a critical baseline comparison for our experiments with centrally-delivered cocaine. Cocaine is traditionally thought to increase extracellular dopamine by acting on the DAT and blocking the reuptake of dopamine (Torres et al., 2003; Cragg and Rice, 2004). DAT blockade could explain both effects observed on evoked-release: as dopamine is released with each stimulation pulse, it is simultaneously cleared from the extracellular space by reuptake. Thus, DAT blockade could account both for the enhanced magnitude of release as well as reduced decay rate.

Lateral ventricular cocaine increased the magnitude of evoked dopamine release without altering decay rate. DAT blockade, therefore, is an insufficient explanation for this result. Rather, since electrical stimulation of the VTA is thought to drive dopamine release, in large part, via glutamate action on dopamine cell bodies (Negus and Miller, 2014), the data suggest that lateral ventricle cocaine enhanced dopamine cell excitability, either through changes to dopamine neu-

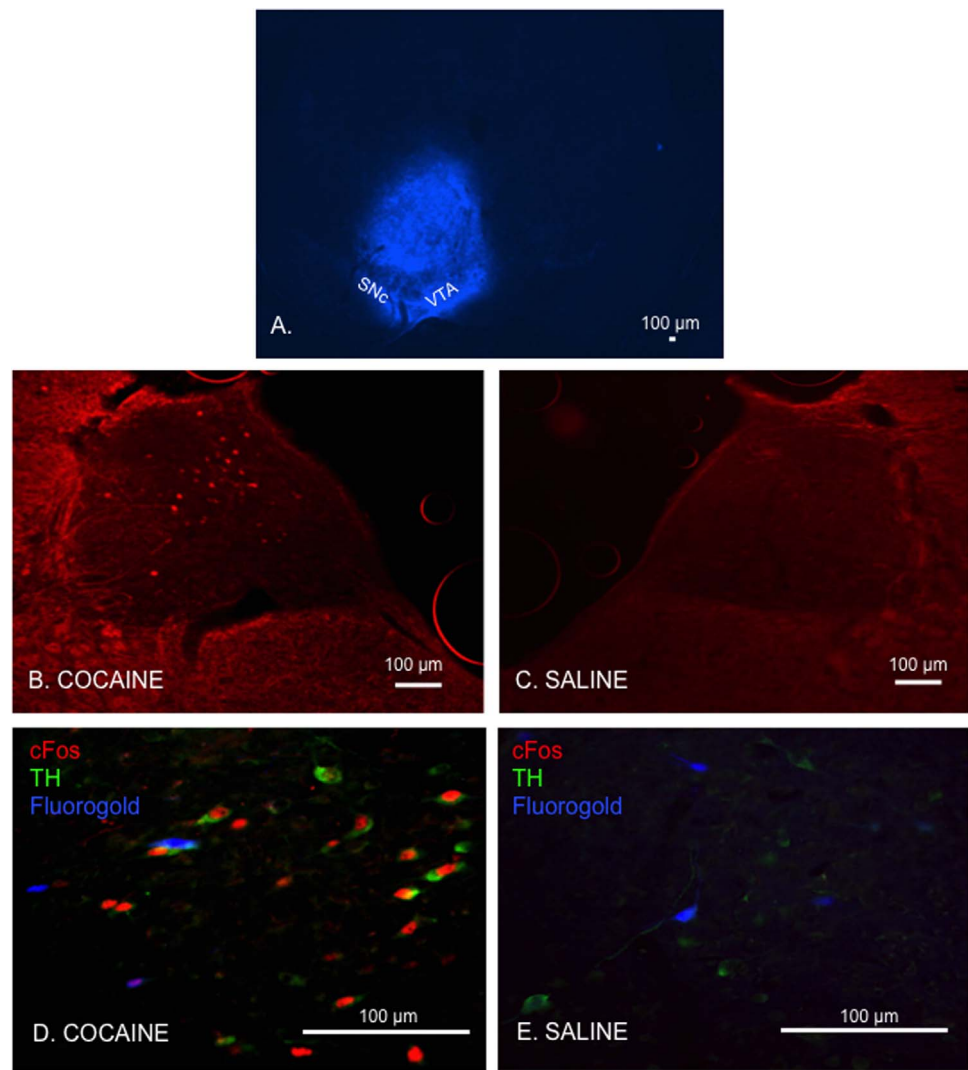


Fig. 5. Fourth ventricular cocaine induces cFos in the NTS. (A). Representative image of FG injection into the VTA. White dashed circles represent the VTA and SNc at the level of -5.2 mm relative to bregma, respectively. (B). Representative image of cFos labeling in the NTS at the level of -13.4 mm relative to bregma of fourth ventricular cocaine-treated rats. (C). Representative image of cFos labeling in the NTS at the level of -13.4 mm relative to bregma of fourth ventricular saline-treated rats. (D). Representative image of colocalization of cFos (red), TH (green), and FG (blue) labeling in the NTS of fourth ventricular cocaine-treated rats. (E). Representative image of colocalization of cFos (red), TH (green), and FG (blue) labeling in the NTS of fourth ventricular saline-treated rats. Horizontal scale bar on each panel illustrates scale.

Table 1
Average cell counts and colocalization percentages represented at each level of the NTS by experimental treatment.

NTS Coordinates	Bregma -14.2 mm		Bregma -13.8 mm		Bregma -13.4 mm	
	Cocaine	Saline	Cocaine	Saline	Cocaine	Saline
cFos	*5.2 (1.0)	0.11 (0.11)	*13.4 (2.2)	0.33 (0.17)	*15.0 (3.1)	1.3 (1.1)
TH	13.9 (1.4)	13.2 (2.4)	13.7 (1.0)	11.5 (1.9)	11.8 (1.5)	11.1 (0.8)
FG	11.3 (1.4)	11.1 (1.4)	9.4 (2.2)	10.2 (1.4)	6.5 (1.2)	7.0 (2.3)
% cFos w/ TH	35.9 (8.6)	11.1 (11.1)	43.2 (4.7)	0 (0)	34.3 (3.7)	1.2 (1.2)
% cFos w/ FG	11.9 (7.0)	0 (0)	5.1 (1.6)	0 (0)	3.2 (1.5)	0 (0)
% cFos w/ TH & FG	5.4 (2.5)	0 (0)	2.6 (0.9)	0 (0)	1.9 (0.9)	0 (0)

Note: Cell count and colocalization averages represented as mean ± SEM.
* = p < 0.05.

rons themselves on an increase in excitatory drive from afferents. Indeed, cocaine increases the burst firing of many VTA dopamine neurons (Koulchitsky et al., 2012; Mejias-Aponte et al., 2015) and rapidly (e.g. within minutes) enhances the excitability of dopamine neurons via direct action on the VTA by enhancing N-methyl-D-aspartate (NMDA) receptor currents (Schilström et al., 2006; Argilli et al., 2008). Cocaine also blocks the norepinephrine (NE) transporter. NE and its receptors mediate a variety of cocaine-stimulated behaviors (Schroeder et al., 2013; Schmidt and Weinshenker, 2014) and are critical mediators of cocaine-induced increases in NAc phasic dopamine signaling (Goertz et al., 2015). Thus, it is possible that lateral ventricular cocaine potentiated evoked dopamine release through direct action within the VTA.

The mechanisms mediating effects of fourth ventricular cocaine are less clear. Fourth ventricular infusions of low volume act on the periventricular hindbrain parenchyma e.g., (Hayes et al., 2009) and thus direct action of fourth ventricular-injected cocaine in the VTA is unlikely. Instead, the data suggest that cocaine activation of hindbrain neurons projecting to the VTA may enhance the excitatory drive to dopamine neurons. Geisler et al. found that systemic cocaine strongly activated VTA-projecting ventromedial mesopontine tegmentum neurons (Geisler et al., 2008). While there was some overlap in cFos

expression with areas that send cholinergic and glutamatergic afferents to the VTA, the overlap was relatively small. Moreover, the exact phenotype of these brainstem-VTA projection neurons could not be identified. However, additional regions of the hindbrain [i.e. parabrachial nucleus (Grabus et al., 2004) and NTS (Grabus et al., 2004; Buffalari and Rinaman, 2014)] that project to the VTA express cFos in response to systemic cocaine. As the NE neurons of the A2 region of the NTS project to the VTA and, as previously mentioned, NE release in the VTA appears critical in mediating cocaine-induced dopamine signaling in the NAc, these neurons are of particular interest.

We explored the possibility that cocaine action in the NTS activates a population of NE neurons projecting to the VTA using immunohistochemistry. Fourth ventricular delivery of cocaine significantly elevated cFos expression in the NTS relative to vehicle delivery. Many of these neurons were positive for TH. However, very few cocaine-activated neurons projected directly to the VTA and even fewer TH-positive neurons projected directly to the VTA. Thus, the cFos data suggest that hindbrain cocaine engages NTS NE neurons, but these neurons likely do not directly potentiate VTA dopamine neuron excitability. Still, the failure to observe cFos in VTA-projecting TH-positive neurons should not be taken as an indication of the absence of a direct projection. cFos immunohistochemistry is used as a proxy for neuronal activation, however there are limitations to this approach. For example, this strategy provides little information about the time course of activation of NTS neurons following fourth ventricular cocaine administration. Nevertheless, our data highlight the NTS as a hindbrain substrate potentially involved in fourth ventricular cocaine's effects on electrically-evoked DA release. The specific mechanisms by which NTS neurons communicate with mid- and forebrain structures to influence dopamine signaling should be investigated in future studies. Recently, NTS neurons that produce glucagon-like peptide 1 (GLP-1) were shown to project directly to the VTA (Alhadeff et al., 2012). GLP-1 has been implicated in modulating cocaine self-administration (Schmidt et al., 2016; Sørensen et al., 2015), cocaine reward (Graham et al., 2013) as well as the excitability of VTA neurons (Mietlicki-Baase et al., 2013). While our data support a role for the caudal brainstem and the NTS specifically in cocaine-potentiated dopamine signaling, identifying cell types and their path to the VTA remains an important future direction.

While cocaine clearly has effects in the periphery (Wise et al., 2008), cocaine must enter the brain to exert effects on dopamine signaling (Porter-Stransky et al., 2011) which, in turn, is thought to be critical for cocaine's reinforcing properties. Our data contribute to a growing recognition that cocaine acts throughout the central nervous system to increase dopamine signaling. Given that fourth ventricular cocaine activates NTS neurons and potentiates evoked dopamine release in the NAc, we now can include the caudal brainstem as another neural substrate upon which cocaine exerts its effects on dopamine signaling. The caudal brainstem and NTS are emerging as a critical node for food reinforcement (Alhadeff and Grill, 2014; Kanoski et al., 2014). The present data extend these findings to suggest that the NTS may also play a role in drug reinforcement through its ability to tune phasic dopamine signaling in the NAc.

4. Experimental procedures

4.1. Subjects

For experiments involving dopamine measurements, forty-two experimentally naïve, male Sprague-Dawley rats (300–400 g at testing, Charles River Laboratories, Chicago, IL), individually housed in plastic cages in a temperature and humidity controlled room on a 12:12 light:dark cycle (lights on at 7:00) were used. Experiments were conducted during the light cycle. Rats were treated according to the guidelines recommended by the Animal Care Committee of the University of Illinois at Chicago. For experiments involving immuno-

histochemistry, male Sprague-Dawley rats (250–265 g upon arrival, Harlan Laboratories, Indianapolis, IN) were individually housed in hanging metal cages within a temperature and humidity controlled room on a 12:12 light:dark cycle. All rats had ad libitum access to pelleted chow (Purina Rodent Chow, 5001) and water. All procedures conformed to and received approval from the institutional animal care and use committees of the University of Pennsylvania.

4.2. Fast-scan cyclic voltammetry

For dopamine recordings, rats were anesthetized with intraperitoneal (IP) urethane (1.5 mg/kg) and prepared for dopamine recordings using fast-scan cyclic voltammetry (FSCV; see Fortin et al., 2015 for details). Using the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 2007) all implants were targeted from bregma. A FSCV guide cannula (Bioanalytical Systems, West Lafayette, IN) was positioned dorsal to the NAc (AP: +1.3 mm, ML: +1.0 mm) and a chlorinated silver (Ag/AgCl) reference electrode was positioned in the contralateral cortex. Rats receiving intracerebroventricular (ICV) infusions were implanted with an infusion cannula directed to either the lateral (AP: –1.0 mm, ML: –3.0 mm, DL: 3.0 mm, angled at 10°) or fourth ventricle (AP: –11.5 mm, ML: 0.0 mm, DL: –6.5 mm). Stainless steel screws were inserted in the skull to secure all implants with dental cement (Safco Dental Supply Company). A carbon fiber microelectrode housed in a custom-made micromanipulator (UIC Machine Shop, Chicago, IL) was then inserted into the guide cannula and the electrode was lowered into the NAc core. Simultaneously, a bipolar stimulating electrode (Plastics One Inc., Roanoke, VA) was lowered just dorsal to the VTA (AP: –5.2 mm, ML: +1 mm, DV: –7.0 mm). A train of current pulses (24 pulses, 60 Hz, 120 μ A, 4 ms/pulse) was administered and the stimulating electrode was lowered in 2 mm increments until dopamine release was evoked and detected at the carbon-fiber electrode in the NAc. Once maximal dopamine release was evoked (DV: –7.5 to –8.5 mm), the stimulating electrode was cemented in place. Rats remained in the stereotaxic frame and experiments were conducted immediately following surgery.

A carbon-fiber recording electrode was loaded into a micromanipulator and connected to a head-mounted voltammetric amplifier attached to a commutator (Crist Instruments, Hagerstown, MD) in a behavioral chamber (Med-Associates, Inc, St Albans City, VT). The voltage of the electrode was held at –0.4 V relative to an Ag/AgCl reference electrode – also connected to the voltammetric amplifier. A triangular waveform of voltage is then applied, and rapidly (400 V/s) scanned from a negative (–0.4 V) to a positive (+1.3 V) potential and back again. The waveform was repeatedly applied at 60 Hz for 20 min, followed by 10 min at 10 Hz in order to equilibrate the recording electrode to maintain a stable background current between scans that can be subtracted out to reveal changes in current due to the oxidation of dopamine over time.

A train of current pulses (24 pulses, 60 Hz, 120 μ A, 4 ms/pulse) was delivered to the VTA every 3 min. The peak oxidative current due to dopamine was measured during the 5 s prior to and the 15 s after the onset of VTA stimulation. Each stimulation evoked a sharp rise in oxidative current followed by an exponential decay. Current was converted to concentration after each electrode was calibrated after use with 1 μ M dopamine in a flow injection system (Sinkala et al., 2012). Peak dopamine concentration ([DA] max) as well as the latency for dopamine concentration to decay from peak concentration by 50% ($t(1/2)$) were determined for each stimulation – the latter being an index of the rate of reuptake by the DAT (España et al., 2008). Once a stable baseline of peak release was achieved – as determined by 3 consecutive recordings within 10% of each other and referred to below as 'baseline' – animals received drug or vehicle either systemically (IP) or centrally to either the lateral or fourth ventricle (ICV). Stimulations continued for 1 h following drug or vehicle administration.

After experiments were completed a stainless steel electrode was

lowered through the guide cannula to the depth of the recording site. Current (1.0 mA, 0.4 s) was applied to create a lesion in order to localize the recording site. Additionally, 1 μ l of India ink (Fischer Scientific) was infused into the infusion cannula to verify placement of either the lateral or fourth ventricle for rats that underwent ICV manipulations. Brains were immediately extracted and stored in 4% formalin for at least 24 h before being mounted and sliced in a -20°C cryostat (LEICA CM1850). Brain slices were mounted on Poly-L-Lysine subbed slides (American Master*Tech Scientific, Inc.) and placements were verified and photographed using bright field microscopy (Olympus BX43 Fluorescence Research Microscope).

Data ([DA]_{max}; t(1/2)) from each rat was expressed as a percentage of the average baseline. Separate statistical comparisons for each of these measures were conducted for each injection/infusion site (systemic, lateral ventricle, fourth ventricle). For each measure within a given injection/infusion site, a 2-way mixed analysis of variance (ANOVA) – where drug treatment (cocaine vs. saline) was a between-subjects variable and time (23 samples) was a within-subjects variable – was calculated. Significant interaction terms were further explored using Bonferroni-corrected *t*-tests at each time point.

4.3. Immunohistochemistry

Rats ($n=9$) received intramuscular anesthesia [ketamine (90 mg/kg; Butler Animal Health Supply, Dublin, OH), xylazine (2.7 mg/kg; Anased, Shenandoah, IA), and acepromazine (0.64 mg/kg; Butler Animal Health Supply)] and subcutaneous analgesia (2.0 mg/kg Metacam; Boehringer Ingelheim Vetmedica, St. Joseph, MO) for all surgeries. Rats were positioned in a stereotaxic device and a unilateral cannula was placed above the VTA (coordinates: 0.6 mm lateral from midline, 5.8 mm posterior to bregma, and 6.4 mm ventral from skull surface, with the injector aimed 2.0 mm below the end of the guide cannulae). While under anesthesia and in the stereotaxic device, animals received a unilateral 200 nl injection of FG in the VTA. Injectors were left in place for 1 min and then removed. A guide cannulae was then placed above the fourth ventricle (coordinates: 2.5 mm anterior to occipital and 5.2 mm ventral to skull on midline) and secured with stainless steel screws and dental cement. FG injection placements in the VTA were verified post-mortem; a representative image of a VTA FG injection is provided in Fig. 5A. Fourth ventricle cannula placements were functionally confirmed via measurement of the sympathoadrenal-mediated glycemic response to 5-thio-D-glucose (210 μ g/2 μ l artificial cerebrospinal fluid) injected into the ventricle as previously reported (Alhadeff et al., 2014; Ritter et al., 1981). A post-injection increase in blood glucose level of at least 100% from baseline was necessary for subject inclusion.

Five days following FG injections, rats were injected in the fourth ventricle with cocaine (50 μ g/1 μ l; $n=6$) or saline ($n=3$). Ninety minutes post-injection, rats were deeply anesthetized and transcardially perfused with 0.1 M pH 7.4 PBS (Boston Bioproducts, Ashland, MA) followed by 4% paraformaldehyde (PFA, Boston Bioproducts). Brains were removed and post-fixed in 4% PFA for 4 h and then stored in 30% sucrose until brains no longer floated. Brains were sectioned coronally (30 μ m) at the level of the NTS and collected serially in triplicate.

Immunohistochemistry (IHC) was conducted according to previous procedures (Alhadeff et al., 2015). Briefly, sections were washed with 1% sodium borohydride followed by washes with 0.1 M PBS. Brain sections were incubated in 5% normal donkey serum for 1 h, followed by an overnight (16 h) incubation with primary antibodies: polyclonal goat anti-cFos primary antibody (1:2,000, sc-52G; Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-tyrosine hydroxylase (TH) primary antibody (1:1,000, 2792S; Cell Signaling, Danvers, MA). Sections were then washed and incubated with secondary antibodies: donkey anti-goat AlexaFluor 594 and donkey anti-rabbit AlexaFluor 488 (1:500, Jackson ImmunoResearch Laboratories, West Grove, PA).

FG autofluorescence was detected using a special filter (C-FL UV-2A; Nikon Instruments, Melville, NY) on a fluorescence microscope (Nikon 80i; Nikon Instruments).

Brain sections were mounted onto glass slides and coverslipped using Fluorogel (Electron Microscopy Sciences; Hatfield, PA). Immunofluorescing neurons were visualized and quantified using fluorescence microscopy (Nikon 80i, NIS Elements AR 3.0) at 10 \times and 20 \times magnification. 8–10 sections per rat were quantified from the NTS [2–4 sections each at approximately -14.2 , -13.8 , and -13.4 mm posterior to bregma]. These three plate levels were chosen as they displayed maximal cocaine-induced cFos expression in the NTS. cFos, FG, and TH cell counts were quantified unilaterally in all experiments, as the vast majority of projections from the NTS to VTA are ipsilateral in nature. Independent samples two-tailed *t*-tests were used to compare counts between injection conditions (cocaine vs. saline) where appropriate.

4.4. Drugs

Cocaine hydrochloride (Sigma-Aldrich, St. Louis, MO) was dissolved into its vehicle (saline, 0.9%) to a concentration of 50.0 μ g/1.0 μ l saline for ICV manipulations. This dose was chosen based on its ability to induce a conditioned place preference when given to the lateral ventricle (Morency and Beninger, 1986). Rats received micro-injections of 1 μ l of this concentration to either the lateral or fourth ventricle. Rats that received systemic (IP) cocaine received a concentration of 2.5 mg/kg body weight dissolved into saline (0.9%). This dose was selected to match the magnitude of effect elicited by ICV cocaine on evoked dopamine release. The monosynaptic retrograde tracer fluorogold (FG, Fluorochrome, LLC, Denver, CO) was dissolved to 2% (w/v) in sterile water.

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